Carcinogenesis vol.21 no.4 pp.607–615, 2000

Breast cancer, heterocyclic aromatic amines from meat and *N*-acetyltransferase 2 genotype

Ralph J.Delfino[1,8], Rashmi Sinha[2], Cynthia Smith[1], John West[3], Edward White[4], Henry J.Lin[5], Shu-Yuan Liao[1,6], Jason S.Y.Gim[5], Hoang L.Ma[5], John Butler[7] and Hoda Anton-Culver[1]

[1]Epidemiology Division, Department of Medicine, College of Medicine, 224 Irvine Hall, University of California, Irvine, CA 92697-7550, [2]Nutritional Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, Rockville, MD, [3]Breast Care Center of Orange, Orange, CA, [4]Saddleback Breast Center, Laguna Hills, CA, [5]Division of Medical Genetics, Harbor-University of California, Los Angeles Medical Center, Torrance, CA, [6]Department of Pathology, College of Medicine, University of California, Irvine, CA and [7]Department of Surgery, College of Medicine, University of California, Irvine, CA, USA

[8]To whom correspondence should be addressed
Email: rdelfino@uci.edu

Breast cancer risk has been hypothesized to increase with exposure to heterocyclic aromatic amines (HAAs) formed from cooking meat at high temperature. HAAs require enzymatic activation to bind to DNA and initiate carcinogenesis. *N*-acetyltransferase 2 (NAT2) enzyme activity may play a role, its rate determined by a polymorphic gene. We examined the effect of *NAT2* genetic polymorphisms on breast cancer risk from exposure to meat by cooking method, doneness and estimated HAA [2-amino-1-methyl-6-phenylimidazole[4,5-*b*]pyridine (PhIP), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (DiMeIQx)] intake. Women were recruited with suspicious breast masses and questionnaire data were collected prior to biopsy to blind subjects and interviewers to diagnoses. For 114 cases with breast cancer and 280 controls with benign breast disease, *NAT2* genotype was determined using allele-specific PCR amplification to detect slow acetylator mutations. HAAs were estimated from interview data on meat type, cooking method and doneness, combined with a quantitative HAA database. Logistic regression models controlled for known risk factors, first including all controls, then 108 with no or low risk (normal breast or no hyperplasia) and finally 149 with high risk (hyperplasia, atypical hyperplasia, complex fibroadenomas). Meat effects were examined within *NAT2* strata to assess interactions. We found no association between *NAT2* and breast cancer. These Californian women ate more white than red meat (control median 46 versus 8 g/day). There were no significant associations of breast cancer with red meat for any doneness. White meat was significantly protective (>67 versus <26 g/day, OR 0.46, 95% CI 0.23–0.94, *P* for trend = 0.02), as was chicken, including well done, pan fried and barbecued chicken. MeIQx and DiMeIQx were not associated with breast cancer. A protective effect of PhIP was confounded after controlling for well done chicken. Results were unchanged using low or high risk controls or dropping 30 *in situ* cases. There was no interaction between *NAT2* and HAAs. These findings do not support a role for HAAs from meat or *NAT2* in the etiology of breast cancer. Further research is needed to explain the white meat association.

Abbreviations: BBQ, barbecued; CI, confidence interval; CIS, carcinoma *in situ*; CYP1A2, cytochrome $P450_{1A2}$; DiMeIQx, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline; HAAs, heterocyclic aromatic amines; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; NAT, *N*-acetyltransferase; OR, odds ratio; PhIP, 2-amino-1-methyl-6-phenylimidazole[4,5-*b*]pyridine; RR, relative risk.

Introduction

Experimental evidence over more than two decades has led to the proposal that heterocyclic aromatic amines (HAAs) may be causal factors in human breast cancer (1). HAAs are formed as a result of cooking meat for long durations by common high temperature methods such as barbecuing, grilling and pan frying. The resulting major HAAs formed at the parts per billion level are 2-amino-1-methyl-6-phenylimidazole[4,5-*b*]pyridine (PhIP), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (DiMeIQx) (2–7). Several HAAs have been found to be carcinogenic in rats and mice in long-term feeding studies, with some causing mammary carcinomas, including MeIQx (8,9) and PhIP (10,11). Mutations of the Ha-*ras* and *p53* genes have been found in PhIP-induced rat mammary carcinomas (12). Evidence for potential genotoxicity is suggested by linear relationships between DNA adduct formation and MeIQx dose in mice at levels comparable with human dietary doses to as low as 1 DNA adduct/10^{11} nucleotides (13). Bioavailability has been demonstrated by findings that MeIQx and PhIP can be detected in the urine of people who eat cooked meats but not in subjects receiving parenteral alimentation (14).

In humans, HAAs require enzymic activation to electrophiles in order to bind to DNA and thus initiate carcinogenesis (15). *N*-acetyltransferase (NAT) activity is important in this regard. NAT transfers acetyl-CoA to the amino (or hydroxyl) side chain of arylamines converting them to unstable electrophiles. This activity has been linked to two genes, referred to as *NAT1* and *NAT2* (16–18). The *NAT2* gene is polymorphic and individuals who carry two allelic mutations have a slow acetylator phenotype, whereas heterozygous wild-type genotypes have an intermediate acetylator phenotype and homozygous wild-type genotypes have a rapid acetylator phenotype (17,19–22). *NAT2* is thought to largely determine whether the acetyltransferase metabolic phenotype of an individual is slow, intermediate or rapid (16,18,23), although *NAT1* may be important as well (23,24). In addition to acetylation a variety of other enzymatic processes, including sulfonation and glucuronidation, are capable of influencing heterocyclic amine metabolism.

The relationship between *NAT2* polymorphisms and breast cancer carcinogenesis is not clearly understood. In the first carcinogen activation step, *N*-oxidation of heterocyclic amines to *N*-hydroxylamines is thought to be catalyzed by cytochrome

$P450_{1A2}$ (CYP1A2) (25). The aryl compounds hydroxylated by CYP1A2 may then be *O*-acetylated by *NAT1*- and *NAT2*-dependent enzymes to *N*-acetoxyarylamine electrophiles in the liver or target organ (26–28). These electrophiles form adducts with DNA (1,27–29) and may be mutagenic (30). An alternative pathway to *N*-acetoxyarylamines is via the *N*,*O*-acetyltransferase activity of *NAT2* (26) or *NAT1* (23). Adducts of HAAs with DNA occur in cultured human mammary epithelial cells, indicating that metabolic activation of HAAs may occur in breast tissue (31).

We hypothesized that due to differences in enzyme activity, polymorphisms in the *NAT2* genotype could increase breast cancer risk when women are exposed to HAAs from meat intake. The objective of the present investigation was to examine breast cancer risk from cooked meat intake and estimated levels of PhIP, MeIQx and DiMeIQx and to evaluate possible interactions with *NAT2* polymorphisms.

Materials and methods

Design and population

A case–control study was conducted on women >39 years old with a suspicious breast mass detected clinically and/or by diagnostic mammography who were scheduled for an open, core or fine needle breast biopsy to rule out mammary carcinoma. Other eligibility criteria included no previous history of cancer, no other severe debilitating medical illnesses and fluent English. Limiting the age of eligibility to 40 and above was intended to achieve a cancer/benign breast disease ratio of no less than 25% (32). Recruited patients had a moderate to high clinical suspicion of a breast carcinoma from mammography and/or other clinical findings sufficient to warrant a diagnostic biopsy. A trained interviewer quickly identified, recruited and interviewed eligible women at one of three participating breast centers: the Breast Care Center of Orange; the Saddleback Breast Center; the UCI Clinical Cancer Center, Department of Surgery. Subjects were interviewed (demographics, family history and meat questionnaire) and had blood drawn at the clinic sites either during a preliminary visit prior to the biopsy date or on the day of biopsy. Prior to the biopsy date, self-administered questionnaires (diet and risk factors) were distributed to 377 of 394 subjects in the present analysis. These subjects were instructed to return them by mail before receiving their diagnosis, which we confirmed, otherwise the data were invalidated to ensure that subjects were blind. It was necessary to have 17 subjects complete questionnaires in the breast center while they waited for diagnostic results.

Cases with malignant tumors and controls with benign masses were then identified histopathologically. Because the incident event is cancer diagnosis, we consider the design to be a pre-incident case–control study. It is intended to reduce many of the case–control biases expected when subjects and researchers are aware of the disease status. It has the potential to reduce participation, recall and interviewer biases. Future investigations are planned to validate these expectations.

Out of 535 patients approached and eligible, 394 participated fully and are included in the present analysis (114 cases and 280 controls) and 86 declined participation. Fifty-five other subjects (partial participants) were excluded from this analysis because they failed to complete all questionnaires prior to receiving diagnostic results ($n = 51$) or refused to supply a blood sample ($n = 4$). The participation rate was similar in patients diagnosed with (82%) and without (85%) cancer.

The institutional review boards of the University of California, Irvine, and the Long Beach Memorial Hospital (for the Saddleback Breast Center) approved the study protocols in accordance with an assurance filed with and approved by the US Department of Health and Human Services. Informed written consent was obtained from all subjects.

Histopathological criteria for case and control subgroup classifications

The study pathologist (S.-Y.L.) conducted a blind review of the slides for patients with benign and malignant diagnoses. Pathological classifications of benign breast disease were made according to Page's criteria (33,34). When sufficient parenchyma adjacent to lesions was available, classifications included non-proliferative disease, proliferative disease without atypia and atypical hyperplasia (35). These histological features were combined with the presence of a fibroadenoma subdivided based upon the presence of either non-complex or complex lesions (sclerosing adenosis or intraductal papilloma). These classifications allowed us to stratify the control groups on low versus high risk of breast cancer. Elevated relative risks (RR) for subsequent development of breast cancer have been identified in cohort studies for women with atypical hyperplasia (RR 2.5–7.3) and women with proliferative disease without atypia (RR 1.6–3.5) (35–39). Low or no risk has been associated in these studies for women without proliferative disease (RR 1.0–1.6). The presence of sclerosing adenosis or intraductal papilloma may confer additional risks (RR 3.10–23.6) (38,40). It is possible that the occurrence of some benign breast disease is due, in part, to risk factors shared with breast cancer cases, risk factors that could include *NAT2* or HAAs. Therefore, separate analyses are reported using three control groups; all controls (280), low risk controls (15 normal breast, 93 no hyperplasia, no atypia) and high risk controls (58 hyperplasia but no atypia, 27 atypical hyperplasia, 39 sclerosing adenosis and 25 intraductal papilloma). Twenty-three controls could not be classified because they underwent only fine needle aspirations or core biopsies and there was insufficient parenchyma surrounding the fibroadenoma. Therefore, they were used only in the analyses including all controls.

Among the cancers, there were 73 invasive ductal carcinomas, 11 invasive lobular carcinomas, 28 ductal and two lobular carcinoma *in situ* (CIS). The 30 women with CIS were put in the case group given their malignant potential for developing invasive cancer (41). Sensitivity analyses were performed to examine changes in results using invasive versus *in situ* cases.

NAT2 genotyping

The *NAT2* genotype was determined using allele-specific PCR amplification (20,22) on DNA extracted from frozen buffy coat samples (42,43). For the present analysis, individuals who were either homozygous or heterozygous for wild-type *NAT2* were classified as fast acetylators, whereas those carrying two slow acetylator mutations were classified as slow acetylators.

Three *NAT2* mutations were assayed for each subject (T341→C, *NAT2**5; G590→A, *NAT2**6; G857→A, *NAT2**7) (44). There were only two black subjects in the study. One was classified as a slow acetylator by the above mutations. The other was tested for a fourth slow acetylator mutation found virtually only among blacks (G191→A, *NAT2**14) (21) and found to have only the wild-type nucleotide. Negative controls were included in every PCR run. A 100% duplicate sample rate was used. The laboratory team was blind to case–control and exposure status.

Dietary nutrients, meat intake and HAA estimates

Dietary nutrients were assessed from the Health Habits and History Questionnaire (HHHQ), a self-administered dietary questionnaire with 112 food items that account for >90% of the intake of major dietary nutrients (45). The nutrient analysis utilized DIETSYS software developed for that questionnaire (46).

Women were asked by trained interviewers about usual meat intake 1 year prior to interview using a questionnaire and color photographs that were developed by Dr R.Sinha at the Nutritional Epidemiology Branch of the Division of Cancer Epidemiology and Genetics, NCI. The questionnaire used was based on the HHHQ approach and is tailored to interface with the DIETSYS software (45,46). Interviewers used color photographs for meat types and doneness levels and flashcards on consumption frequency and portion size by cooking method for each meat type. Grams daily intake for specific meats by doneness and cooking method were computed by multiplying estimated portion size by reported frequency of consumption. Red meats included hamburger patty/cheeseburger, beef steak, pork chops, bacon and breakfast sausage. White meats included chicken (or turkey) and fish. Fish questions did not include canned fish (e.g. tuna) or shellfish. For each meat the method of cooking was assessed and 10 associated frequency categories were given, including never, <1/month, 1/month, 2–3/month, 1/week, 2/week, 3–4/week, 5–6/week, 1/day, 2/day and ⩾2/day. Cooking methods included pan fried, deep fat fried or fast food (for chicken, fish), grilled/barbecued (BBQ), fast food hamburgers, oven broiled, baked/roasted and microwaved. Intake of meat gravy from meat dripping was also assessed. Doneness photos were used for hamburger/cheeseburger, beef steak (four photographs, rare, medium, well done, very well done), pork, bacon (three photographs, rare, medium, well done), BBQ chicken, pan fried chicken (three photographs, just done, well done, very well done). Intermediate preferences could be chosen by subjects and coded, e.g. medium rare. Doneness for breakfast sausage was also assessed by flashcard (just done, well done, charred).

The intake of HAAs were estimated by multiplying the gram intake of each meat type from the questionnaire (including doneness level and cooking method) by the HAA concentration in a HAA database for the corresponding meat type. Estimated levels of intake for the HAAs were summed across all meat items to give a total intake of MeIQx, DiMeIQx and PhIP for each subject. This approach is described in detail elsewhere (5,6,47).

Statistical analysis

Statistical significance for descriptive case–control comparisons was attributed to two-sided P values <0.05 from Wilcoxon rank sum tests for continuous variables and from logistic regression for categorical variables adjusted for age. Multivariate logistic regression models (48) were used to examine the

effects of meat intake, estimated HAA intake and *NAT2* genotype on the risk of breast cancer to estimate odds ratios (ORs) and 95% confidence intervals (CIs). Models were tested for risk factors that may confound and/or modify the effects of interest, including age at diagnosis, age at menarche, menopausal status, age at first full-term pregnancy, parity, months of pregnancy, lactation history (months breast feeding), education level, race/ethnicity, family history of breast cancer in first and second degree relatives and body mass index (kg weight/m^2 height). Subjects were considered post-menopausal if they reported cessation of menstruation over 6 months ago and were over 49 years old. Subjects under 50 years old were considered post-menopausal if they reported natural menopause or bilateral oöphorectomy.

Meat variables included intakes (g/day) of total red meat, total white meat and specific meat types by cooking methods known to increase HAAs (pan fried, BBQ or grilled and increasing levels of doneness). For regression models, chicken and the sum of red meats were examined by cooking method and doneness. Other dietary factors were also examined for potential confounding effects, including total energy intake (kcal/day), total fat (g/day), percent of calories from fat, total protein (g/day) and total fruit and vegetables (servings/day). Risks from meat intake and HAAs were analyzed as continuous variables and as categorical variables based upon the distribution of exposure in all controls (with lower quartiles or tertiles as the referent category). Logistic regression analyses of continuous variables were used to test for trend in ordinal exposure–response relationships.

The effects of the meat variables and *NAT2* genotype on the risk of breast cancer were first assessed separately in bivariate models and then in models controlling for confounding variables. A confounding effect was assumed if parameter estimates changed by at least 10%. To assess the possibility that the effects of HAAs differ in fast versus slow acetylators, PhIP, MeIQx and DiMeIQx were examined within the two *NAT2* strata. Assessments of multiplicative interactions between *NAT2* genotype and HAAs were also evaluated in adjusted logistic regression models. The fit of the model was assessed with the likelihood ratio test.

Results

Subject characteristics and dietary intake

Demographic and reproductive characteristics are presented in Table I. Cases are significantly older than controls for all case–control comparisons. Post-menopausal women were more likely to be cases than pre-menopausal women, but the difference was not significant using high risk controls. Although there are proportionally more cases whose age at first full term pregnancy was 25–29 years old as compared with the referent group of <25 years, older groups and nulliparous women were not at increased risk. The other reproductive factors were not significant. There is no case–control difference in body mass index. Both cases and controls were mostly educated beyond high school and there were no significant case–control differences. There is a suggestion of an increased risk of breast cancer among women with a family history of breast cancer in the mother or a sister, but the difference is not significant ($P = 0.17$). However, there is a significant increased risk of breast cancer with a positive family history in a second degree relative (grandmother or aunt).

Table II compares the dietary intake of cases versus controls. There are no significant differences between cases and controls in the intake of calories, fat or fruits and vegetables, but there is a suggestion of higher protein intake in high risk controls versus cases (49 versus 43 g/day, $P = 0.08$). Both low and high risk control groups ate more meat than cases and the difference was dominated by white meat ($P = 0.0007$). The only significant difference for red meat intake was a higher consumption of grilled or BBQ red meat among low risk controls versus cases (median 2.8 versus 0.0 g/day, respectively, $P = 0.04$). Only 49 cases (43%) ate any grilled or BBQ red meat and only 53 cases (46%) ate any grilled or BBQ chicken, which explains the medians of 0.0 presented in Table II. Both cases and controls ate considerably more white meat than red meat (4.8 and 5.6 times more, respectively). The higher consumption of white meat among controls versus cases is due to chicken intake ($P = 0.0002$), not fish ($P = 1.0$). This significant difference is seen in comparisons of cases with both low and high risk controls, with somewhat greater differences using high risk controls. The higher intake in controls was also seen for grilled and BBQ chicken, but not pan fried chicken (mean 1.1 g/day for cases versus 1.2 g/day for controls). The mean intake of deep fat fried chicken (not shown) was also low and not different between cases (0.6) and controls (0.6 g/day). For BBQ and pan fried chicken combined (not shown), most women ate chicken well done, which was the only doneness category significantly higher in controls (mean 2.9 g/day for cases versus 7.2 g/day for controls, $P = 0.006$). Fewer preferred it just done (mean 3.1 g/day for cases versus 2.8 g/day for controls, $P = 0.24$) or very well done (mean 1.1 g/day for cases versus 1.7 g/day for controls, $P = 0.88$). Table II also shows that controls had a notably greater estimated intake of PhIP than cases (92 versus 61 ng/day, $P = 0.004$), which is seen for both low and high risk controls. There was no significant difference in MeIQx, but controls had a higher intake of DiMeIQx.

Given the low intake of grilled, BBQ and pan fried chicken, these meat types were combined for the regression analysis of effects of well done chicken. Because the medians for this variable in cases and control groups were 0, cut-points for categorical comparisons were 0 for the lowest category and the 75th percentile in controls (9.6 g/day) was used as the cut-point for the intermediate (3.2–9.6 g/day) and high categories (>9.6 g/day). Other chicken, total white meat and HAA analytical variables were defined at the quartiles of control distributions. Given the low intake of red meat, categorical variables were defined at the tertiles of control distributions.

Breast cancer risk from NAT2 genotype, meat and estimated HAA

The population was predominantly white, non-Hispanic (92%). The frequency of slow acetylator mutations was less in 15 Asian subjects (33%) than in 361 non-Hispanic white subjects (59%), consistent with population estimates (20). Among 16 Hispanics the frequency was 62% and one of two African-Americans was a slow acetylator. Regression models were retested with whites alone and results were not altered. Therefore, models presented include all subjects.

There are no significant associations between breast cancer risk and *NAT2* genotype regardless of control group used (Table III). There was no difference in the distribution of slow *NAT2* by cancer histopathology (61–64% ductal and lobular invasive carcinomas and CIS; not shown).

Table IV shows multivariate-adjusted logistic regression models for meat and HAAs adjusted for age, menopausal status and family history of breast cancer. Addition of other reproductive and demographic variables did not improve the fit of the models or confound parameters for meat or HAAs. Dose–response relationships are suggested for both white meat and chicken not fried or BBQ. All ORs for the highest versus lowest quartiles are <0.5, with upper 95% CI <1.0, except for white meat in models using low risk controls. The strongest protective effects are in models using high risk controls. The highest versus lowest tertile for well done pan fried and BBQ chicken is also significantly protective and the ORs are <0.5. The intermediate tertile is not protective. Red meat intake is not significantly associated with breast cancer. The upper tertile compared with lower tertile for BBQ and pan fried red

Table I. Demographic and reproductive characteristics in case and control subjects

Characteristic	Cases (n = 114)	All controls (n = 280)	P value[a]	Low risk controls (n = 108)	P value	High risk controls (n = 149)	P value
Age (years, mean ± SD)	61 (12)	54 (10)	0.0001	52 (10)	0.0001	54 (10)	0.0001
Menopausal status (no. and %)							
Pre-menopausal (referent)	28 (25)	113 (40)		53 (49)		49 (33)	
Post-menopausal	86 (75)	167 (60)	0.004	55 (51)	0.0002	100 (67)	0.15
Age at menarche (years, mean ± SD)	13 (1.3)	13 (1.5)	0.83	13 (1.5)	0.89	13 (1.5)	0.62
Age at first full term pregnancy (no. and %)							
<25 years old (referent)	45 (39)	126 (45)		47 (44)		69 (46)	
25–29 years old	30 (26)	47 (17)	0.09	13 (12)	0.03	29 (20)	0.23
>29 years old	11 (10)	41 (15)	0.55	22 (20)	0.44	17 (11)	0.90
Nulliparous	28 (25)	66 (23)	0.68	26 (24)	0.79	34 (23)	0.67
Parity (no. and %)							
Nulliparous (referent)	28 (25)	66 (23)		26 (24)		34 (23)	
1–2 children	54 (47)	145 (52)	0.98	58 (54)	0.91	77 (52)	0.97
>2 children	32 (28)	69 (25)	0.92	24 (22)	0.85	38 (25)	0.86
Months pregnancy (mean ± SD)	19 (14)	18 (14)	0.52	16 (12)	0.26	18 (15)	0.83
Months breast feeding (mean ± SD)	2.9 (5.4)	3.6 (6.3)	0.38	3.3 (5.9)	0.98	3.8 (6.8)	0.33
BMI (kg/m^2, mean ± SD)	25.1 (5.0)	24.9 (5.3)	0.59	24.8 (6.2)	0.28	25.0 (4.8)	0.99
Education (no. and %)							
High school or less (referent)	26 (23)	44 (16)		16 (15)		27 (18)	
College or vocational	60 (53)	172 (61)	0.31	65 (60)	0.39	90 (60)	0.84
Post-graduate/professional	28 (24)	64 (23)	0.76	27 (25)	0.98	32 (22)	0.30
Family history of breast cancer (no. and %)[b]							
None (referent)	65 (58)	182 (66)		71 (67)		93 (64)	
Mother or sister	23 (20)	47 (17)	0.17	19 (18)	0.25	27 (18)	0.37
Grandmother or aunt	25 (22)	46 (17)	0.02	16 (15)	0.03	26 (18)	0.10

[a]Two-sided P values for continuous variables are from Wilcoxon rank sum tests and for categorical variables they are from unconditional logistic regression as compared to referent categories, adjusted for age. Menopausal status is not adjusted for age.
[b]The family history of one case and five controls is unknown.

Table II. Dietary and heterocyclic aromatic amine intake[a] of case and control subjects (median and 25th and 75th percentiles)

Variable	Cases (n = 114)	All controls (n = 280)	P value[b]	Low risk controls (n = 108)	P value	High risk controls (n = 149)	P value
Total calories (kcal/day)	979 (790–1301)	1078 (772–1447)	0.20	1078 (744–1462)	0.28	1056 (799–1407)	0.25
Total fat (g/day)	38 (25–55)	39 (32–57)	0.50	39 (26–59)	0.39	39 (25–54)	0.70
Total protein (g/day)	43 (32–57)	47 (34–63)	0.13	44 (33–63)	0.54	49 (36–62)	0.08
Total fruits and vegetables (servings/day)	3.1 (2.3–4.8)	3.3 (2.1–4.6)	0.75	3.1 (1.9–5.0)	0.53	3.3 (2.1–4.5)	0.67
Total meat (g/day)	44.1 (25.4–63.5)	59.1 (36.9–81.2)	0.0001	56.8 (34.4–77.2)	0.01	60.1 (39.5–85.9)	0.0001
Red meat (g/day)	6.6 (1.6–14.3)	8.3 (0.0–18)	0.20	8.3 (2.6–20.1)	0.19	7.5 (0.0–17.9)	0.36
Pan-fried	1.8 (0.0–5.2)	2.8 (0.0–7.0)	0.22	1.8 (0.0–7.0)	0.49	2.8 (0.0–7.0)	0.17
Grilled/BBQ	0.0 (0.0–5.7)	0.0 (0.0–7.5)	0.10	2.8 (0.0–9.2)	0.04	0.0 (0.0–7.0)	0.35
Rare/medium done	2.8 (0.0–9.2)	3.3 (0.0–10.7)	0.25	3.7 (0.0–11.7)	0.16	2.8 (0.0–10.3)	0.50
Well/very well done	0.5 (0.0–3.7)	1.6 (0.0–7.3)	0.13	2.3 (0.0–7.4)	0.14	1.3 (0.0–7.2)	0.22
White meat (g/day)	31.5 (13.7–53.0)	46.2 (25.7–67.4)	0.0007	40.1 (25.7–59.9)	0.04	51.3 (27.4–69.5)	0.0001
Fish	2.8 (0.0–12.1)	2.8 (0.0–12.1)	1.0	2.8 (0.0–12.1)	0.30	5.7 (0.0–13.1)	0.54
Chicken total	25.6 (11.3–44.3)	38.7 (20.1–53.0)	0.0002	30.3(17.0–53.0)	0.008	44.8 (25.6–541)	0.0001
Chicken grilled/BBQ	0.0 (0.0–9.6)	3.2 (0–16.0)	0.03	3.2 (0.0–16.0)	0.01	3.2 (0.0–13.7)	0.11
Chicken pan fried	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.44	0.0 (0.0–0.0)	0.70	0.0 (0.0–0.0)	0.35
PhIP (ng/day)	61 (7–131)	92 (30–240)	0.004	91 (30–203)	0.03	103 (30–241)	0.004
MeIQx (ng/day)	7.2 (3.1–19.0)	9.7 (3.6–22.6)	0.19	10.5 (3.7–22.0)	0.24	9.2 (3.9–24.2)	0.22
DiMeIQx (ng/day)	0.6 (0.0–1.7)	0.9 (0.1–2.5)	0.03	1.0 (0.1–2.7)	0.03	0.9 (0.0–2.5)	0.07

[a]Heterocyclic aromatic amine intake was estimated from consumption of specific meats, cooking method and doneness preferences.
[b]Two-sided P values are from Wilcoxon rank sum tests of case versus control consumption.

meat cooked well to very well done is nearly significant (all controls and low risk controls) to significant (high risk controls). Co-regression of red meat with chicken variables or total dietary protein with meat variables did not alter the above associations.

Table IV also shows that subjects with estimated intakes of PhIP in the highest (>240 ng/day) versus the lowest quartile (<31 ng/day) are at significantly decreased risk of breast cancer (OR 0.42, 95% CI 0.20–0.88). Parameters for MeIQx and DiMeIQx were also negative, but not significant. None of the trend tests were significant. Also, Spearman's rank correlation between PhIP and well done pan fried and BBQ chicken

Table III. *N*-Acetyltransferase 2 (*NAT2*) genetic polymorphisms and risk of breast cancer

NAT2 genotype	Cases (no. and %)	All controls[a] (no. and %)	OR (95% CI)[b]	Low risk controls[c] (no. and %)	OR (95% CI)	High risk controls[d] (no. and %)	OR (95% CI)	Total (no. and %)
Fast acetylators	44 (39)	121 (43)	1.00 (referent)	49 (45)	1.00 (referent)	61 (41)	1.00 (referent)	165 (42)
WT/WT	7	23		8		13		30
WT/T341C	27	56		25		24		83
WT/G590A	10	39		15		22		49
WT/G857A	0	3		1		2		3
Slow acetylators	70 (61)	159 (57)	1.24 (0.78–1.97)	59 (55)	1.39 (0.78–2.48)	88 (59)	1.10 (0.65–1.86)	229 (58)
T341C/T341C	18	44		19		23		62
T341C/G590A	38	88		31		51		126
T341C/G857A	3	5		1		2		8
G590A/G590A	9	18		7		10		27
G590A/G857A	1	2		0		2		3
G857A/G857A	1	2		1		0		3

WT, wild-type allele lacking the slow acetylator mutations T341C, G590A and G857A.
[a]Includes 23 women with insufficient tissue from benign breast disease biopsy to classify the degree of cellular proliferation for low and high risk classification.
[b]From unconditional logistic regression models adjusted for age.
[c]Normal breast or benign breast disease histopathologies with non-proliferative changes.
[d]Benign breast disease histopathologies of hyperplasia with no atypia, atypical hyperplasia or complex fibroadenomas (sclerosing adenosis, intraductal papilloma).

was 0.53 ($P < 0.0001$). To test whether the finding for PhIP was due to chicken intake, PhIP was co-regressed with well done chicken. This led to an increase in the OR for the upper quartile of PhIP from 0.42 to 0.61 (95% CI, 0.27–1.38), but virtually no change in the OR for the upper quartile of chicken (0.45 to 0.47, 95% CI, 0.21–1.06). Co-regression of continuous scaled PhIP and chicken variables led to a 26% decrease in the parameter estimate for PhIP ($P = 0.3$), but the estimate for chicken was reduced only 3% and still significant ($P = 0.02$). These findings show that the PhIP association is confounded by chicken.

The above models were also tested with smoking variables (never versus former versus current, cigarettes/day and years smoked). None of the smoking variables were significant and there was no confounding of effects for meat intake or HAAs.

Testing models stratified by acetylator genotype yielded no evidence that *NAT2* genotype modifies the effects of HAAs since all ORs for upper versus lower quartiles were <1.0 for both fast and slow acetylators, consistent with findings for combined groups shown in Table IV. Most models showed upper confidence limits well above 1.0. Among fast acetylators, ORs for PhIP second, third and fourth quartiles as compared with the lowest quartile were 0.37, 0.66 and 0.58, respectively, and for slow acetylators the ORs were 0.68, 0.95 and 0.31, respectively. For PhIP in the slow acetylator group, the upper quartile for PhIP gave the only significant OR, but again, the association was confounded by chicken intake. Models for multiplicative interaction between *NAT2* genotype and HAAs using all subjects are also non-significant ($P = 0.6$ for PhIP, $P = 0.3$ for MeIQx and DiMeIQx).

Separate models for pre- and post-menopausal women were consistent with the above models in Table IV for effects of meat and HAAs, but a small sample size in the pre-menopausal group led to non-significant ORs. For white meat, ORs for the second, third and fourth quartiles as compared with the first quartile were 0.71, 0.60 and 0.58, respectively, for post-menopausal women (P for trend = 0.13) and 1.12, 0.54 and 0.36 for pre-menopausal women (P for trend = 0.12).

Models were also retested for cases with invasive breast cancer alone, excluding the 30 *in situ* cases. Overall, ORs were unchanged, but upper 95% confidence intervals included or slightly exceeded 1.0 due to decreased sample size. For instance, the OR for the upper versus lower quartile was 0.45 (95% CI 0.20–1.00) for white meat intake and was 0.52 (95% CI 0.24–1.12) for chicken not fried or BBQ.

Discussion

Overview of findings

The average intake of the three HAAs estimated from foods measured in a USDA sponsored random survey of 3563 people from the US population showed concentrations of PhIP >> MeIQx > DiMeIQx (7), which is consistent with the present study. Also, the frequency of slow acetylator mutations we found was consistent with population estimates (20).

We found no independent associations between *NAT2* and breast cancer risk. Although one study found that all seven subjects with lobular (in contrast to ductal) invasive breast cancers had a rapid *NAT2* genotype (49), we found no difference in *NAT2* by cancer histopathology. Also, intake of red or white meat cooked by methods known to increase HAAs, as well as estimated exposure to HAAs, did not increase breast cancer risk. On the contrary, due to a protective effect of white meat, and particularly chicken intake, associations were inverse for all cooking methods and for HAA exposure. Furthermore, there was no clear divergence of effects after subgroup analyses in fast versus slow acetylators and no statistical interactions between *NAT2* genotype and HAAs.

The P value for trend is significant to nearly significant for a protective effect of well/very well done red meat (Table IV). However, the sample size of the highest risk group is small, suggesting that this result may be spurious. Below, we relate this finding to alternative explanations.

Comparisons with the literature

None of the epidemiological studies examining *NAT2* genetic polymorphisms has found it to be independently associated

Table IV. The relationship between breast cancer risk and meat intake and heterocyclic aromatic amine intake from meat

Independent variable	Cases[a] (no.)	All controls (no.)	OR (95% CI)[b]	Low risk controls[c] (no.)	OR (95% CI)	High risk controls[d] (no.)	OR (95% CI)
Meat							
White meat							
<26 g/day	44	68	1.00 (referent)	26	1.00 (referent)	31	1.00 (referent)
26–46 g/day	31	69	0.80 (0.43–1.46)	32	0.63 (0.29–1.34)	33	0.80 (0.39–1.63)
47–67 g/day	22	69	0.55 (0.29–1.05)	25	0.47 (0.21–1.09)	41	0.48 (0.23–0.99)
>67 g/day	16	69	0.46 (0.23–0.94)	23	0.46 (0.19–1.11)	41	0.36 (0.17–0.78)
Trend test[e]			$P = 0.02$		$P = 0.11$		$P = 0.006$
Chicken not fried or BBQ							
<8 g/day	40	71	1.00 (referent)	35	1.00 (referent)	28	1.00 (referent)
8–26 g/day	29	63	0.62 (0.30–1.28)	24	0.90 (0.36–2.27)	32	0.48 (0.20–1.13)
27–42 g/day	25	71	0.58 (0.29–1.15)	23	0.63 (0.26–1.50)	43	0.44 (0.20–0.99)
>42 g/day	19	70	0.37 (0.20–0.68)	24	0.45 (0.21–0.96)	43	0.27 (0.13–0.54)
Trend test			$P = 0.11$		$P = 0.27$		$P = 0.04$
Well done pan fried and BBQ chicken							
0 g/day	82	168	1.00 (referent)	65	1.00 (referent)	88	1.00 (referent)
3.2–9.6 g/day	20	42	1.20 (0.64–2.26)	14	1.41 (0.62–3.23)	23	1.15 (0.56–2.34)
>9.6 g/day	11	65	0.45 (0.22–0.91)	27	0.37 (0.16–0.85)	35	0.46 (0.21–0.99)
Trend test			$P = 0.02$		$P = 0.01$		$P = 0.02$
Red meat							
<3.0 g/day	42	92	1.00 (referent)	31	1.00 (referent)	53	1.00 (referent)
3.0–15 g/day	47	90	1.10 (0.64–1.89)	36	0.92 (0.46–1.83)	46	1.16 (0.63–2.14)
>15 g/day	24	93	0.57 (0.31–1.04)	39	0.46 (0.21–0.97)	47	0.63 (0.32–1.24)
Trend test			$P = 0.12$		$P = 0.25$		$P = 0.15$
Red meat rare/medium done							
<3.3 g/day	68	137	1.00 (referent)	48	1.00 (referent)	78	1.00 (referent)
3.3–8.5 g/day	16	58	0.64 (0.33–1.24)	26	0.47 (0.21–1.03)	27	0.78 (0.37–1.64)
>8.5 g/day	29	80	0.78 (0.45–1.35)	32	0.75 (0.38–1.48)	41	0.90 (0.49–1.68)
Trend test			$P = 0.58$		$P = 0.75$		$P = 0.76$
Red meat well/very well done							
<1.6 g/day	63	138	1.00 (referent)	49	1.00 (referent)	77	1.00 (referent)
1.6–6.7 g/day	28	60	1.00 (0.56–1.77)	25	0.82 (0.40–1.68)	29	1.07 (0.55–2.07)
>6.7 g/day	22	77	0.58 (0.32–1.06)	32	0.47 (0.23–0.98)	40	0.56 (0.29–1.10)
Trend test			$P = 0.06$		$P = 0.09$		$P = 0.05$
Heterocyclic aromatic amines							
PhIP							
<31 ng/day	47	69	1.00 (referent)	26	1.00 (referent)	38	1.00 (referent)
31–92 ng/day	23	69	0.54 (0.29–1.03)	28	0.57 (0.26–1.27)	32	0.61 (0.30–1.27)
92–240 ng/day	30	72	0.84 (0.46–1.54)	30	0.78 (0.37–1.68)	39	0.92 (0.46–1.84)
>240 ng/day	13	65	0.42 (0.20–0.88)	22	0.53 (0.22–1.32)	37	0.38 (0.17–0.86)
Trend test[e]			0.20		0.52		0.20
MeIQx							
<3.6 ng/day	32	69	1.00 (referent)	25	1.00 (referent)	36	1.00 (referent)
3.6–9.7 ng/day	33	69	1.03 (0.55–1.92)	24	1.22 (0.54–2.76)	41	0.81 (0.40–1.65)
9.8–22.6 ng/day	26	68	0.97 (0.50–1.86)	32	0.80 (0.36–1.78)	31	1.03 (0.49–2.18)
>22.6 ng/day	22	69	0.66 (0.34–1.31)	25	0.66 (0.28–1.54)	38	0.55 (0.26–1.19)
Trend test			0.13		0.24		0.08
DiMeIQx							
<0.1 ng/day	32	74	1.00 (referent)	26	1.00 (referent)	43	1.00 (referent)
0.1–0.9 ng/day	39	64	1.43 (0.77–2.64)	26	1.27 (0.58–2.76)	31	1.57 (0.78–3.16)
1.0–2.5 ng/day	28	69	1.05 (0.56–2.00)	26	0.94 (0.42–1.12)	37	1.11 (0.54–2.25)
>2.5 ng/day	14	68	0.53 (0.25–1.10)	28	0.43 (0.17–1.04)	35	0.50 (0.22–1.15)
Trend test			0.14		0.14		0.11

[a]Includes 23 women with insufficient tissue from benign breast disease biopsy to classify the degree of cellular proliferation for low and high risk classification.
[b]From unconditional logistic regression models adjusted for age, menopausal status and family history of breast cancer in first and second degree relatives.
[c]Normal breast or benign breast disease histopathologies with non-proliferative changes.
[d]Benign breast disease histopathologies of hyperplasia with no atypia, atypical hyperplasia or complex fibroadenomas (sclerosing adenosis, intraductal papilloma).
[e]*P* value for trend is from adjusted logistic regression models of continuous meat and HAA variables.

with breast cancer risk (49–52). One showed increased risk of breast cancer from smoking in slow acetylators (50) while others showed some limited smoking effects among fast acetylators, but no dose–response relationship (51,52). Only Millikan *et al.* (52) evaluated breast cancer risk from *NAT1* genotype and did not find any independent associations. Nevertheless, experimental evidence suggests *NAT1* may be important at target sites in the human mammary gland (53).

Three recent studies examined the relationship of breast cancer risk to meat intake by degree of doneness (54–56). A

case–control analysis from the Iowa Women's Health Study examined effects of meat intake by doneness preference using the same photographs as used in this study for red meat, but none for chicken (54). Consumption patterns were very different from the present population, with higher median red than white meat intake (30 versus 24 g/day in controls, respectively, and 36 versus 24 g/day in cases, respectively). Women who usually consumed hamburgers, beef steak and bacon very well done compared with women preferring rare to medium doneness showed an OR of 4.62 (95% CI, 1.36–15.7). However, there was no dose–response relationship for actual intake of well to very well done red meat because the ratio of ORs comparing the highest to lowest tertile of intake was 1.17. This suggests either notable misclassification of reported intake or case–control recall bias (57).

Only two studies examined breast cancer risk from meat intake and *NAT2* genotype (55,56). In an analysis of the Nurses Health Study cohort, there were no reported associations of breast cancer risk with red meat intake or red meat cooking method (including high temperature methods and charring) and no evidence for interaction with *NAT2* genotype (55). A case–control study found that red meat intake was not associated with breast cancer risk and risk for meat variables did not vary by *NAT2* genotype (56). There were no data on doneness. The same study reported decreased risks among women with increasing intakes of poultry, fish or pork. Premenopausal women in the highest versus lowest quartile of poultry intake (>43 versus <19 g/day) were at significantly decreased risk (OR 0.6, 95% CI 0.4–0.9) and post-menopausal women were similarly protected (P for trend = 0.01). Combining poultry intake variables for the present study, we also found a significant inverse trend ($P = 0.02$). Women in the highest versus lowest quartile of poultry intake (>53 versus <21 g/day) were at decreased risk (OR 0.46, 95% CI 0.24–0.90).

The protective effect of white meat consumption remains unexplained. It may be that the amino acid content of white meat supports proper immune function, thereby enhancing tumor surveillance at higher levels of intake. The tendency for increased intake of total protein (Table II) and red meats (Tables II, IV) in controls versus cases supports this speculation because controls have a better overall protein intake. An increasing intake of white over red meat may also be a surrogate for a variety of health-conscious behaviors that overall lowers risk. It is unlikely that decreases in saturated fat from meat explains the protective effect given a lack of convincing evidence in the literature on dietary fat and breast cancer risk (58). We found no effect of saturated fat ($P = 0.5$).

Strengths and limitations

Because eligible patients, participants and interviewers were essentially blind to the true diagnoses, the present design has the potential to reduce participation, recall and interviewer biases. Interviewing patients prior to diagnosis minimizes or eliminates both the physiological effects of treatment and influences of health-related information on the perception of lifestyle behaviors such as diet. Also, controls underwent breast cancer detection similar to cases and were selected under similar conditions. The present design shares the advantages of incident over prevalent case–control studies because all diagnosed cases were invited to participate regardless of duration of disease or treatment success and recall of information prior to diagnosis was enhanced.

Some of the negative results, particularly for gene–environment interactions, could be due to a sample size insufficient to detect small magnitude effects. It is also possible that *NAT2* is a risk factor for benign breast disease in the same manner that it is a risk factor for breast cancer. This would bias relationships toward the null hypothesis. The only study to compare acetylator phenotype between benign breast disease and healthy controls found non-significant differences for cystic disease with epithelial hyperplasia versus normal controls (43.8 versus 55.2% slow acetylators, respectively) and for 'cystic disease' alone (42.6 versus 55.2% slow acetylators, respectively) but no differences for 'fibroadenoma' alone (59). It is also possible that HAAs from well done meat act as a positive risk factor for benign breast disease as well as for breast cancer, thereby biasing the relationship toward the null hypothesis. However, for relationships of breast cancer risk to both *NAT2* and the meat variables, we found little change in parameter estimates after restricting the analysis to the group having lower risk benign breast disease and normal breast tissue. Furthermore, the protective effects of white meat were enhanced in models using high risk controls. This is not what would be expected if meat intake played a shared role in both benign breast disease and cancer.

Most of the patients in this study were recruited from breast centers serving largely white well-educated women. Therefore, the present findings may not be valid for other populations of poorer women or women in other racial/ethnic groups. Also, given the low intake of red meat, results may differ in populations eating higher amounts of red meat. However, it has been shown that for PhIP, which is the HAA with the highest concentration in the American diet, concentrations are higher in chicken than in red meat cooked well to very well done using high temperature methods (47).

The present findings may be more applicable to women in California and other US regions with less traditional dietary preferences. Dietary trends in California towards healthier diets, including less fat and red meat, is evidenced by a proliferation of health food supermarkets and juice bars and by a long-standing California Department of Health Services program promoting increased intake of fruits and vegetables that was later adopted by the NCI (60). Only one subject was a vegetarian, who had no measurable influence on estimates of effect.

Data from the self-administered diet questionnaire we used for estimating major dietary nutrients (45) yielded an apparent underestimation of intake, especially calories. This is possibly due to under-reporting, but it is more likely that women in this geographic region are eating foods not in the questionnaire. The current version of the HHHQ (not available at the start of the present study) now accounts for many of these foods. Nevertheless, the diet data are probably sufficient to rank subjects by their food intake.

An additional weakness of the dietary questionnaires is the focus on determining patterns of intake for only the previous year. This does not enable the estimation of intake patterns during periods of exposure dating back to several years ago, which are likely to be relevant for the early initiating events in breast cancer. This is particularly important given the selection of women over the age of 39. Lifestyle patterns with regard to meat intake have changed considerably over the past decade, particularly in California. A cohort study design with repeated measures would address these weaknesses because

recall bias is particularly problematical for distant recall in case–control studies.

Implications

Given the results of the three recently reported studies cited above (54–56) and the present investigation, it is important that the public do not get the wrong message from the scientific literature on mammary cancer and HAAs. The only medical advice concerning meat doneness that is proven correct is that there are health risks from undercooking meat related to known infectious diseases. Cooking meat adequately is still important advice despite recent epidemiological evidence that well done red meat is associated with colorectal adenomas (61) and stomach cancer (62). Other research has shown no association between cancers of the colon, rectum, bladder and kidney and estimated HAAs from interviews using photographs of meat cooked to different degrees (63).

Given the experimental evidence linking HAAs to mammary carcinogenesis, the biological plausibility of an adverse effect of HAAs on breast cancer risk remains, but, to date, this has not been demonstrated in human populations. Nevertheless, certain host characteristics, inherited or acquired, could enhance the potential for mammary carcinogenesis in women exposed to HAAs in meat. For instance, other research has suggested the importance of detoxifying enzyme systems. Null genotypes (absence of enzyme activity) for one of these enzyme systems (glutathione *S*-transferases M1, P1 and T1) has recently been associated with breast cancer risk (64). Other dietary factors may also alter host response to HAAs. In a study by Roberts-Thomson and Snyderwine (65), the frequency of PhIP-induced mammary gland tumors was higher in Sprague–Dawley rats fed high fat diets than in those fed low fat diets, although the trend in Ha-*ras* somatic mutations was the reverse. We tested this model with our data by examining the effects of the HAAs in women with $>38\%$ of their calories from fat versus those at $\leqslant 38\%$. There was no suggestion of effect modification, with both groups showing ORs <1.0 for HAAs.

Given the low intake of red meat in the present study population, it is conceivable that other populations with higher red meat intakes may be at increased risk of breast cancer from higher concentrations of HAAs, as well as other putative carcinogens on cooked meat surfaces, such as benzo[*a*]pyrene. It is also possible that a high threshold exists that is rare in most populations relying on meat as their main source of protein and that the threshold may be lowered somewhat by some combination of host, metabolic or other genetic susceptibilities.

Acknowledgements

We wish to thank our research assistants for their diligent efforts at data collection and processing: Jane Curtin, Nutritional Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute; Irene Masunaka, Randy Williams, Presley Hayes and Tara McKittrick, Epidemiology Division, Department of Medicine, University of California, Irvine. This research was supported by funds from the University of California Breast Cancer Research Program, grant no. 1RB-0295. H.J.L. was supported by NIH grants 1R01CA66782 and 1R01CA73403.

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Received June 18, 1999; revised November 18, 1999; accepted November 29, 1999